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Characterization Of A Putative Glycinergic Nigropallidal Pathway In The Rodent Brain

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CHARACTERIZATION OF A PUTATIVE GLYCINERGIC NIGROPALLIDAL PATHWAY IN THE RODENT BRAIN

by

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DEDICATION

My biggest motivation in life has always been my parents' support system. Their strong work ethic served as a daily example to be followed. The high academic expectations placed upon me as a child served as a driving force that impelled me to excel and pursue a graduate degree. With great honor and respect, I would like to dedicate my thesis to my beloved parents, Luis Ortega and Maria Esther Ortega. It is thanks to their unconditional love and care that I have been able to achieve my goals. Because of them, I am proud of the person whom I have become. Their teachings, morals and values will forever be instilled in me, and I can only hope to one day follow in their footsteps as a parent.

To my brother and sister, my lifelong friends, thank you for your guidance and care. I will forever cherish the countless laughs and memories that we have shared. I want to also thank my brother-in-law, Hector, whom I love like a brother, for always lending a helping hand when I have been in need. And to my nieces and nephews, who have completed our family and filled it with joy and happiness.

Finally, I want to present this achievement to my husband, Carlos Escobar, my best friend. Thank you for demonstrating to me that true kindness still exists in this world. Your bright outlook in life inspires me daily and has made me a better person.

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THESIS

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ABSTRACT

Glycinergic neurotransmission is essential for the function of sensory and motor systems associated with pain transmission and rhythmic motor patterns. Yet, the way glycinergic circuits modulates motor systems via the globus pallidus external (GPe) is not clear. The cyto-architecture of glycinergic circuitry is poorly understood and most work has focused in brainstem areas. It is believed that glycinergic neurons are mainly interneurons regulating local circuits. However, it is now becoming clear that they are also projection neurons, constituting true neuronal circuits. At the glycinergic synapse, termination of neurotransmission and recycling of glycine is regulated by two transporters, glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2). GlyT1 and GlyT2 are both expressed in the cerebellum, brainstem and throughout the spinal cord. In addition to those areas, GlyT1 is expressed in subcortical forebrain regions and the midbrain. GlyTs are essential for survival and knockout of either gene leads to animal death shortly after birth, due to impaired breathing and muscular spasm. Based on our preliminary results, glycinergic neurons expressing GlyT1 are found within structures of the basal ganglia, a brain area involved in somatosensory integration, voluntary motor movement and an array of diverse cognitive functions. Preliminary results also demonstrate that GlyT1 immunoreactivity co-localize with GAD67, suggesting a dual phenotype, GABAergic and glycinergic. Interestingly, the physiological role of these neurons containing and releasing both inhibitory neurotransmitters remains unknown and deserves further investigation. Thus, the overarching hypothesis is that these putative glycinergic neurons help modulate critical basal ganglia function via regulation of a novel nigropallidal pathway. To test this hypothesis, we used fluorescent dyes and viral tracing techniques to outline glycinergic neuronal projections to the GPe. Given the role of the GPe in voluntary motor movement, the results from this project have significant potential to inform the development of new therapeutic approaches for basal ganglia-related disorders such as Parkinson's disease (PD) and Huntington's chorea.

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CHAPTER 1

A. BACKGROUND AND SIGNIFICANCE

Background

A1. Glycine Transporters

The neurotransmitter glycine regulates an array of coordinated motor and sensory functions such as breathing, several aspects of vision, audition and voluntary motor activity (2,9, 33,37,39). For decades, glycinergic neurons were believed to be restricted to caudal regions of the central nervous system (CNS) including the spinal cord, brain stem and cerebellum. Interestingly, more recent studies demonstrate that glycine-related neurotransmission involving GlyT1 is widespread, and in addition to the caudal CNS, it is present in many subcortical areas of the forebrain (20,35). However, the precise location of the cell bodies, the projections and connections are still not defined for most neural circuits. Hence, the inhibitory role of glycinergic neurotransmission within areas such as the basal ganglia remains to be determined. Decoding the inherent control of glycinergic neurons upon the GPe can pave the way to a better understanding of basal ganglia dysfunction and resultant pathology. During inhibitory glycinergic neurotransmission, glycine contained in synaptic vesicles is released by presynaptic Ca^{++}-mediated influx to further bind to postsynaptic glycine receptors (GlyR), which are ligand-gated chloride channels. Activation of the GlyR leads to hyperpolarization of the postsynaptic neurons and inhibition of transmission of an action potential. Termination of inhibitory neurotransmission is achieved by re-uptake of glycine into the presynaptic neurons by the action of two Na+,Cl--dependent glycine transporters (GlyT1 and GlyT2) (2,6,12). It is worth mentioning that GlyT1 and GlyT2 are essential for survival and they appear to be involved in complementary rather than the same functions. It has been proposed that GlyT2 is neuronal and GlyT1 glial; however, more recent published findings demonstrate the presence of GlyT1 in neurons, suggesting two distinct

population of glycinergic neurons (6,12,18,33,35). Additionally, glycine functions as an excitatory neurotransmitter by serving as a co-agonist with glutamate for N-methyl-D-aspartate receptor (NMDAR) activation, and the availability of glycine is tightly controlled by GlyT1 activity (2,4). Thus, these transporters may serve as critical pharmacological targets in the treatment of various neurological disorders, ranging from inhibition of chronic pain transmission to potentiation of NMDAR activation in Schizophrenia and Bipolar disorder (20). Therefore, pharmacological inhibition of the activity of GlyT1 and/or GlyT2 is clearly implicated in treatment of several CNS disorders.

To differentiate the precise localization of glycinergic cell bodies and projections in forebrain areas and investigate the function of these neurons, we aimed to elucidate the cyto-architecture of glycinergic projections in the globus pallidus external (GPe), which is a key brain area involved in the regulation of voluntary movement and motor-controlled cognitive functions and reward (22,23,24). This research project is relevant to better understand multiple neurological conditions involving motor and cognitive functions.

A2. Pharmacology

In recent years, GlyTs have emerged as important pharmacological targets in the treatment of neurological disorders. For example, screening of GlyT1 blockers has demonstrated to be a potential pharmacological therapeutic for schizophrenia, based in the hypothesis that the negative symptoms of schizophrenia are originated from hypo-function of the NMDA receptor. Bitopertin (RG1678, La Roche) was among the new class of GlyT1 inhibitors that reached Phase III clinical trials, but unfortunately failed to improve some negative symptoms such as withdrawal and lack of motivation (19,20). Our preliminary results suggest that a large amount of GlyT1-immunoreactivity in the midbrain is contained within glycinergic neurons rather than associated with excitatory glutamate neurons or glial cells, probably accounting for the side-effects in the clinical trials. The findings in preliminary studies demonstrate strong co-localization of GlyT1 with

GAD67-positive neurons, suggesting a dual phenotype of this neuronal circuit, GABAergic and glycinergic. This study will help clarify the connectivity and functions in which glycinergic circuitry participates within the basal ganglia, and thus its involvement in inhibitory neurotransmission, along with GABA. These findings suggest that a better characterization of the localization and identification of GlyT1 containing cells will help explain the multiple effects of drugs that target GlyT1. This project is designed to shed light into the fine innervation and function of these GlyT1-containing glycinergic cells. A better understanding of the location, direction and connection of glycinergic circuits will likely open new venues for pharmacological therapeutics and their role in sensory and motor functions.

A3. Significance

The gathered data may help establish the presence and function of glycinergic circuitry within the basal ganglia as well as the potential effects of dysfunctional and irregular glycinergic neurotransmission in sensory-motor, motor and cognitive capabilities. It is critical to identify glycinergic projections found in the GPe, as these may trigger a cascade of altered feedforward signals that can influence basal ganglia pathways and resultant motor behaviors. Several lines of evidence point to these glycinergic neurons as a novel, undescribed circuit within the basal ganglia: (1) Our preliminary studies indicate that GlyT1 immunoreactivity in the GPe is comprised of nerve fibers and devoid of cell bodies. (2) Injection of the retrograde tracer FluoroGold (FG) in the GPe labeled cell bodies, NeuN-positive, in the substantia nigra pars compacta (SNc), suggesting that the SNc is the location of glycinergic cell bodies. (3) Glycinergic immunoreactivity in the GPe and SNc co-localize with GAD67-positive neurons, suggesting that these are inhibitory neurons with a dual phenotype: GABAergic and glycinergic. (4) Glycinergic terminals in the GPe appear juxtaposed to postsynaptic neurons positive for the glycine receptor a3 subunit. To date, there is no single study describing these glycinergic nigropallidal neurons, demonstrating the novelty of this study. The simultaneous co-release of glycine and GABA has been detected in

regions including the nucleus tractus solitarii (NTS) of the rat as well as in the avian superior olivary nucleus. Therefore, the dual expression of the gamma-aminobutyric acid receptor (GABAR), particularly $GABA_A$ receptor, and GlyR are found postsynaptically (6,9,11). The dual release of GABA and glycine has been demonstrated as these are both transported in the vesicular inhibitory amino acid transporter (VIAAT) (6,34,38). Studies suggest this corelease might help enhance and fine tune inhibitory neurotransmission, as miniature inhibitory post synaptic currents (mIPSCs) occur at different rates between GABA and glycine, since GABA elicits slower mIPSCs (1,11).

The preliminary findings suggest that this research will expose the contribution of glycinergic fibers to complex basal ganglia-dependent behaviors. We will delineate neuronal projections by expression of mCherry carried in AAV-derived particles. These results will provide evidence of the location of the glycinergic nuclei as well as projections and nerve terminals. The experimental design may help correlate glycinergic transmission to basal ganglia-regulated behaviors.

A4. Signaling in the basal ganglia

Introduction. Glycinergic neurons expressing GlyT1 and GlyT2 are known to be primarily present in caudal areas of the central nervous system, where glycine acts as the primary inhibitory neurotransmitter (6,12). However, the literature fails to provide data that establishes the location of glycinergic cell bodies and axonal innervations throughout the basal ganglia that modulate sensory and motor functions. In terms of motor control, the basal ganglia is composed of interconnected nuclei located in the striatum, GPe, globus pallidus internal (GPi), subthalamic nucleus, and the substantia nigra (10,16,21) (Figure 1.2). Although there are multiple structures that partake in the modulation of movement, the basal ganglia serves to integrate information deriving from the parietal, frontal, and temporal cortex. Particularly, glutamatergic electrical stimulation from the cortex to the striatum, derives from regions implicated in the planning and

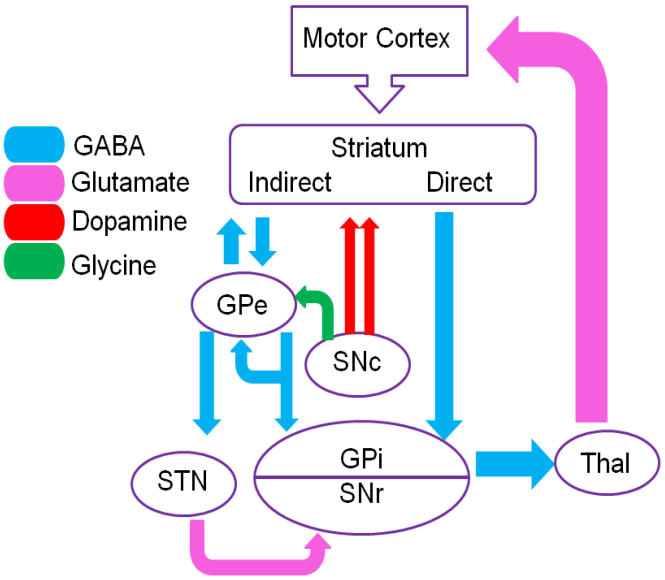

Figure 1.1 Projections of the Basal Ganglia. Nigrostratial dopaminergic projections modulate the striatonigral (direct) and striatopallidal (indirect) pathways. Glycinergic projections of the SNc help modulate the GP. GABAergic projections of the GP innervate all the nuclei within the basal ganglia.

execution of motor movement (28,31). This information activates GABAergic medium spiny neurons in the striatum (16,17). Of note, GABA is ubiquitously found in the central nervous system and serves as the main inhibitory neurotransmitter. GABAergic projections deriving from the striatum modulate the striatonigral (direct) and striatopallidal (indirect) pathways (Figure 1.1). These two pathways result in ideal synchronization and proper regulation of basal ganglia-dependent motor activity (7,22,30). Signaling within the direct and indirect pathways occur via interconnections of GABAergic, glutamatergic and dopaminergic networks. These inhibitory and excitatory mechanisms are the underlying system by which the feedforward and feedback circuitry is regulated (17,21,22). Dopaminergic innervations from the SNc can have either excitatory or inhibitory effects in motor control via D1 and D2 receptors and thereby elicit activation of the striatonigral or striatopallidal pathway, respectively. In turn, these two pathways regulate and balance basal ganglia-related motor movement (8,17) (Fig. 1.1). Importantly, GABAergic projections from the striatum to the GPe denote the initiation of the striatopallidal pathway, where the GPe acts as the main input structure. The GABAergic projections of the GPe innervate all the nuclei within the basal ganglia (Figure 1.1). Therefore, the GPe acts as a relay station that mediates proper regulation of the striatopallidal pathway and integration center for the direct and indirect pathways (14,16,22). Inhibitory GPe projections directly innervates the GPi and the subthalamic nucleus, which are considered the primary target

structures. However, the role of the GPe in the striatopallidal pathway is to ultimately control the output structures of the basal ganglia which include the substantia nigra pars reticulata (SNr) and the GPi, which project to the brainstem and thalamus (Figure 1.2) (16,21,22). Therefore, the GPe is important for proper modulation of the signaling cascade within the indirect pathway that ultimately modulates signal transduction from the thalamus to motor association regions in the cortex (16,22,23), in addition to GABAergic efferents projecting from the SNr to motor nuclei of the brainstem (21). Dysregulation of GPe inhibitory output will cause irregular signaling downstream the striatopallidal pathway that will potentially lead to deficits in basal ganglia-dependent motor movement. Additionally, the GPe helps modulate dopaminergic neuronal signaling of the SNc, as electrical stimulation deriving from the pallidus inhibits pars compacta activity through GABAA receptors. It has been demonstrated that altered activity of pallidal neurons evoke irregular signaling patterns of dopaminergic neurons, which in turn may cause a shift in signaling rates to the striatum and its activating role of the direct and indirect pathways

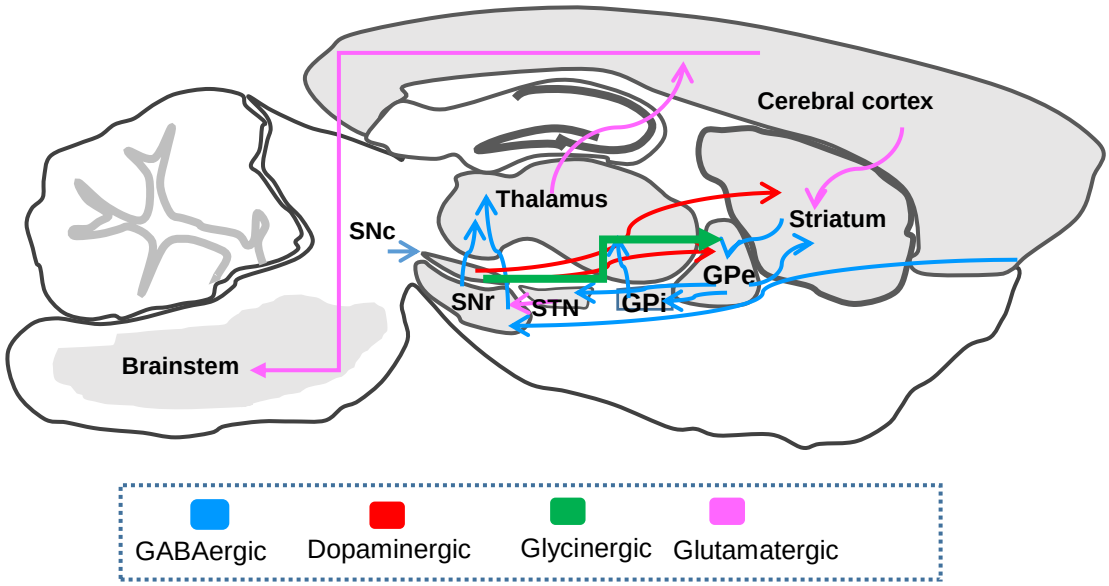

Figure 1.2 The Cortico-striato-pallido-thalamic Loop. The basal ganglia is composed of interconnected nuclei located in the striatum, globus pallidus (GP), entopeduncular nucleus (globus pallidus internal in humans), subthalamic nucleus, and the substantia nigra.
Outline traced from the Allen online Atlas.

(16,17). Interestingly, axonal collaterals from the SNc also provide innervation to the GPe through dopaminergic signaling (3,26). The literature suggests that activation of dopaminergic receptors in the GPe play an important regulatory role of GABAergic activity. Therefore, stimulation of dopaminergic receptors is also involved in the modulation of pallidal neural firing. The depletion of dopaminergic stimulation in the GPe suggests a potential role in PD motor deficits and correlation with symptoms such as tremors and dyskinesia (3,7).

The rationale for this study is to localize and delineate glycinergic circuitry within the basal ganglia and better understand its role in motor movement regulation.

B. SPECIFIC AIMS

Aim 1: Identification of glycinergic circuitry containing GlyT1 in the basal ganglia using retrograde and anterograde tracing approaches. Hypothesis: GlyT1 immunoreactivity in the SNc corresponds to neurons and represents a novel circuit within the basal ganglia. Several neuronal circuits in the basal ganglia and the neurotransmitters being released are well defined. By contrast, the presence of glycine or glycinergic cells has not been considered as a component of the basal ganglia. Therefore, our objective is to identify the cell bodies, the direction of the projections and connections of these glycinergic neurons. These experiments will elucidate novel pathways that may influence the synchronized modulation of the basal ganglia. Two separate experimental designs will be performed to investigate this aim:

a. Retrograde tracing of projections innervating the globus pallidus external using the fluorescent dye FluoroGold (FG), and thus localization of the corresponding cell bodies of glycinergic projections.
b. Circuit delineation of nigropallidal neurons using the anterograde tracer Dextran Red and via Adeno Associated Viral (AAV) particles carrying the reporter gene encoding for mCherry.

CHAPTER 2

MATERIALS AND METHODS

Perfusions. The animal was sedated with an intraperitoneal (IP) injection containing a lethal dose (1.5 mg/kg) of sodium pentobarbital (650 mg/ml; Pentobarbital sodium salt C-IIN, Lot #SLBG3215V, Sigma). Once unconscious and reflex-free, a v-shaped incision was made under the diaphragm, across the torso, and a hemostat was used to clamp the xinophoid process and expose the heart (Illustration 2). First, the descending aorta was clamped. An incision was then made on the left ventricle and a needle was introduced into the aorta. Low-pressure forceps were used to clamp the needle in place. The transcardial perfusion was then performed using a pump (MasterFlex console drive, Cole-7Parmer Instrument Company, IL). 0.9% saline solution was pumped until the liquid streaming through was clear and no longer contained blood. As the saline solution began to flow through, a snip was made in the right atrium to release the liquid accumulation. After being exsanguinated, the tissue was fixated with 500ml of 4% paraformaldehyde (Paraformaldehyde Granular, Cat. No. 19210, Electron Microscopy Sciences, Hatfield, PA) in 1X phosphate buffered solution (PBS; 137 mM Sodium Chloride, Lot. # 081M0051V, Sigma-Aldrich, St. Louis, MO; 2.7 mM potassium chloride, ACS reagent, 99.0-100.5%, P3911, Sigma; 8.1 mM sodium phosphate dibasic, SigmaUltra, minimum 99.0%, S7907, Sigma; 1.47 mM potassium phosphate monobasic, crystal, 7100-12, Mallinckrodt Chemicals, Phillipsburg, NJ). Next, the brain was extracted and post-fixed in 4% PFA for 24 hours at 4°C. Finally, 15 mls of 30% sucrose (S7903, Sigma) in 1X PBS were used to cryoprotect the brain for 24 hours at 4°C.

Tissue collection. A microtome (Leica SM2000R) was used to section the brain sagittally. An ice platform was first built on the stage using potassium phosphate buffered saline (KPBS; 0.02 M

Table 1. Nissl Procedure	
Duration	**Solution**
3 minutes per solution	DI Water, 50%, 70%, 95%, 95%, 100%, 100%, 100% EtOH
5 min.	Xylenes 1
30 min.	Xylenes 2
2 min. per solution	Xylenes 1, 100%, 100%, 100%, 95%, 95%, 70%, 50% EtOH, DI Water
3-10 dips	Nissl Stain
20 dips	DI Water, 50%, 70%
3 min. per solution	95%, 95%, 100%, 100%, 100% EtOH, Xylenes 1, Xylenes 2

potassium phosphate, 150 mM NaCl). The KPBS was leveled and smoothed out. The brain was fixed on the ice platform onto its right hemisphere, and a frozen layer of KPBS was built around it to immobilize it. The tissues were sliced 30μm thick and were collected in 24-well plates, 5 tissues per well, containing antifreeze kryoprotectant (6.58 mM Sodium Phosphate Monobasic Anhydrous, Cat. No. 195500, MP Biomedicals, LLC, Solon, Ohio; 19.23 mM Sodium Phosphate dibasic, Sigma; 30% Ethylene glycol, anhydrous, 99.8%, 324558, Sigma-Aldrich; 20% Glycerol, G6279, Sigma-Aldrich, St. Louis, MO). The 24-well plates were stored at -20°C.

Nissl Staining: One tissue per well was subjected to Nissl staining and used to define and identify specific structures of the target areas. Particularly, to visualize the site of injection and precise location of glycinergic cell bodies. To do so, 10X mosaic images of the Nissl-stained tissues were attained using the Zeiss Observer, AxioVision program. The mosaic images were then compared to the sections in “The Rat Brain” atlas. Prior to Nissl staining, VWR Micro Slides (Superfrost® Plus, Cat. No. 48311-703, 25 X 75 X 1 mm, VWR International, LLC, Randor, PA) were gelatin-coated. The slides were first subbed in Alconox (242985, Sigma) overnight. The next day, distilled water was used to rinse the slides for 2-3 hours. Once dried, they were submerged for 5 minutes in 1% Knox Original Gelatin Unflavored solution (Kraft Foods Global, Inc., Northfield, IL) at 45°C and left to dry in a 65°C oven for 2-3 days. Fresh thionine staining solution (5.84% glacial acetic acid A490-w12, Fisher Chemicals, Fair Lawn, NJ; 1M sodium hydroxide, S318, Fisher Scientific; 0.5% Thionine acetate A18912, Alfa Aesar, Heysham, Lanks, UK) was simultaneously prepared. To begin, 6 tissues were mounted per gelatin-coated slide and were left to dry overnight before being subjected to Nissl staining. The procedure, as seen on **Table 1,** was carefully timed. First,

the slides were subbed in Milllipore water, followed by ethanol (CDA 19, histological grade, A406P, Fisher Scientific). The ethanol solutions were diluted at different concentrations (50%, 70%, 95%, 95%, 95%, 100%, 100%, 100% EtOH), which were used to dehydrate and rehydrate the tissue, respectively. 100% Xylenes (histological grade, 534056, Sigma-Aldrich) solutions were used to remove lipids from the tissue. Once completed, the slides were left to dry for 3-4 hours at room temperature. Lastly, 350 µl of DPX mountant for histology (06522, Sigma) was used to coverslip the slides with a Gold Seal® Cover Glass (24 X 60, No.1, reorder # 3323) and were left to dry for 24-48 hours.

Immunohistochemistry assays: The FluoroGold-containing sections were subjected to dual immunostaining using glycinergic and neuronal markers. The GlyT1 rabbit (Rb) primary antibody was used in combination with a second primary antibody in immunohistochemistry assays. Dilutions varied in accordance to the antibody being used. To remove all antifreeze residue, Tris-buffered solution (1X TBS: 19.98 mM Trizma® base, T1503, Sigma; 149.9 mM sodium chloride; pH 7.5) was used to wash the tissue sections in 24-well plates. Of note, all washes were done at room temperature on a belly dancer shaker (The Belly Dancer, 115V, Alkani Scientific Inc., Pompano Beach, FL) and consisted of 5 consecutive cycles of 5 minutes each. Subsequently, the tissues were incubated at room temperature for 2 hours in blocking solution (2% Goat Serum, G9023, Sigma-Aldrich; 0.1% Triton X-100, Sigma; in 1X TBS). After the blocking step, the primary antibody incubation lasted for 20 hours at 4°C, on a shaker and covered with foil paper. All primary antibodies were diluted in blocking solution (Table 2). Immediately after, the tissues were washed with 1X TBS solution. The tissues were then incubated in the secondary antibody solution for 2 hours, at room temperature and covered with foil paper. The secondary antibodies were diluted in 1X TBS (Table 3). Lastly, the tissues were once more washed in 1X TBS and mounted on VWR micro slides and were left to dry for 2 hours in the dark. They were ultimately coversliped with sodium bicarbonate glycerol (300 µl; 80% glycerol, sigma; 20 mM sodium bicarbonate, sigma). A

double coat of clear nail polish was applied around the edges to seal the coverslip. The acquired images were retrieved using both an inverse (Zeiss Observer 1) and confocal microscope.

Table 2. Primary Antibodies

Name	**Manufacture**	**Host Species**	**Lot#**	**Cat#**	**Dilution**
GlyT1	Dr. L Girotto-Gentil	Rabbit	In-House G080	In House	1:1000
Mouse X Neuronal Nuclei (NeuN)	Millipore	Mouse	NG1876252	MAB377	1:1000
Anti-GAD67, Clone 1G10.2 (Mouse Monoclonal)	Millipore	Mouse	2090450	MAB5406	1:500
Mouse Anti-MAP2 a&b Monoclonal antibody	Millipore	Mouse	LV1486527	MAB378	1:1000
Anti-Tyrosine Hydroxylase, Clone LNC1	Millipore	Mouse	2003001	MAB318	1:500
Mouse monoclonal [GF5] to GFAP-astrocyte marker	Abcam inc.	Mouse	N/A	10062	1:1000

Table 3. Secondary Antibodies

Name	**Manufacture**	**Anti-Species**	**Lot#**	**Cat#**	**Dilution**
Cy™3-conjugated AffiniPure Donkey Anti-Rabbit IgG	Jackson ImmunoResearch Laboratories, Inc.	Rabbit	76381	711-165-152	1:5000

Alexa Fluor® 488-conjugated AffiniPure Donkey Anti-mouse IgG	Jackson ImmunoResearch Laboratories, Inc.	Mouse	104332	715-545-151	1:2000
Cy™3-conjugated AffiniPure Anti-Mouse IgG	Jackson ImmunoResearch Laboratory, Inc.	Mouse	N/A	N/A	1:5000
Alexa Fluor® 488-conjugated AffiniPure Donkey Anti-Rabbit IgG	Jackson ImmunoResearch Laboratory, Inc.	Rabbit	103947	711545152	1:2500

CHAPTER 3

D. RESULTS

Glycine acts as the main inhibitory neurotransmitter in caudal regions of the CNS, including the spinal cord, brain stem and cerebellum (2,6). Consistent with this idea, we found that GLYT2 glycinergic neurons were restricted to caudal areas of the CNS (Fig.2A). Immunoreactivity demonstrated that GlyT1 is expressed in the same regions as GlyT2; however, GlyT1 is more dispersed and it is detected throughout the midbrain (Fig. 2B).

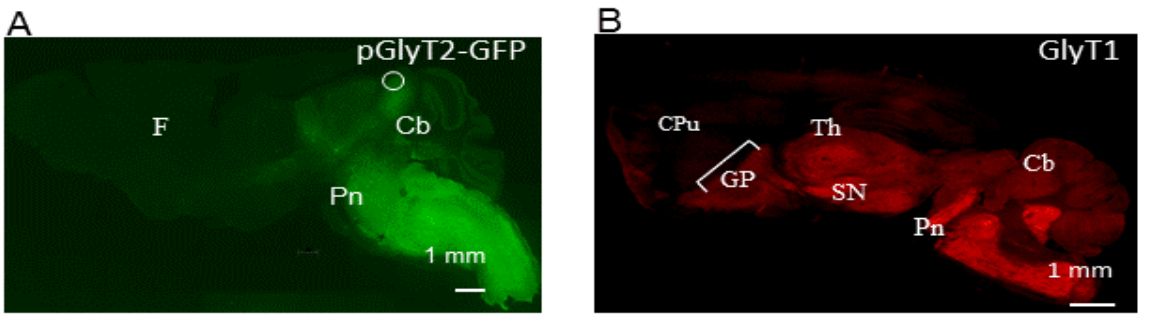

Fig. 2| Expression of GlyT1 and GlyT2 in the central nervous system A) Expression of GlyT2-GFP B) Immunoreactivity of GlyT1.

D.1 Immunoreactivity of GlyT1 in the central nervous system. To characterize GlyT1 expression in the forebrain and midbrain, we subjected brain tissue to immunostaining with a highly specific GlyT1 antibody, followed by fluorescence microscopy analysis. We observed dense immunoreactivity in the midbrain, thalamus, and GP, and complete absence in the striatum. To have a better outlook on the organization of GlyT1 throughout the basal ganglia, particularly in the SNc, we compared the staining with markers for GABAergic and dopaminergic neurons, neuronal cell bodies and astrocytes. As shown in Fig. 3, immunostaining with GlyT1 and the glial fibrillar acidic protein (GFAP) demonstrate that GlyT1 is not highly contained in astrocytes, but

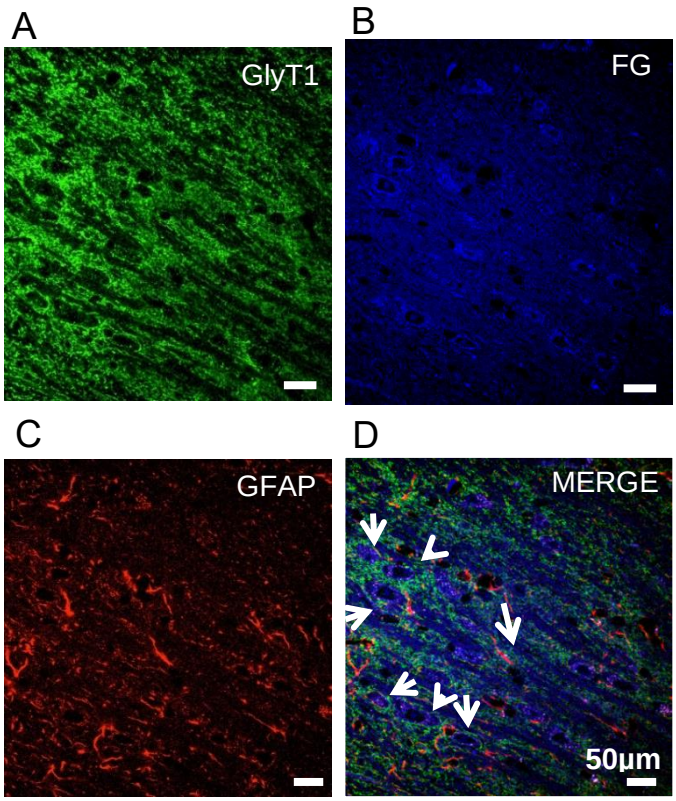

Fig. 3| 63X Confocal images of GlyT1, FG and GFAP. A) GlyT1 fluorescence in the SNc B) Neuronal cell bodies labeled with FG in the SNc C) Astrocytes labeled with GFAP in SNc D) Merged image. Arrows demonstrate the different distribution of GlyT1 between astrocytic fibers and FG labeled neuronal cell bodies.

rather in the membranes of the surrounding neurons. GlyT1 seems to co-localize with microtubule-associated protein 2-positive neurons (MAP2) in the SNc (Fig. 4). These results suggest that GlyT1 immunoreactivity in the SNc and other forebrain areas correspond to glycinergic neurons. Additional immunostaining assays with glutamic acid decarboxylase (GAD67) showed reactivity throughout the brain, including the cortex, hippocampus, striatum, and dense signal in the brain stem and the GP (Fig. 5). Interestingly, GlyT1 immunoreactivity in the GP showed a complete overlap with GAD67, suggesting that these neurons may be GABAergic and glycinergic. Similar phenotype GABA/glycine has been reported in brain stem areas (11). Confocal microscopy imaging of neurons in the SNc indicate a strong co-localization of GlyT1 and GAD67 (Fig. 6). Tissue staining was carried out to establish the location of neuronal cell bodies labeled with FG that contained GlyT1. Microscopic images demonstrated that glycinergic neuronal cell bodies are in the SNc (Fig 7B). Further staining of FG containing sections with tyrosine hydroxylase (TH) antibodies showed that both, glycinergic and dopaminergic neuronal projections originate in the midbrain, join the medial forebrain bundle to terminate in two different anterior areas of the forebrain. Interestingly, a separation of nerve terminals can be observed as glycinergic fibers innervate the GP but not the striatum. By contrast, dopaminergic fibers continue its course through the GP to finally

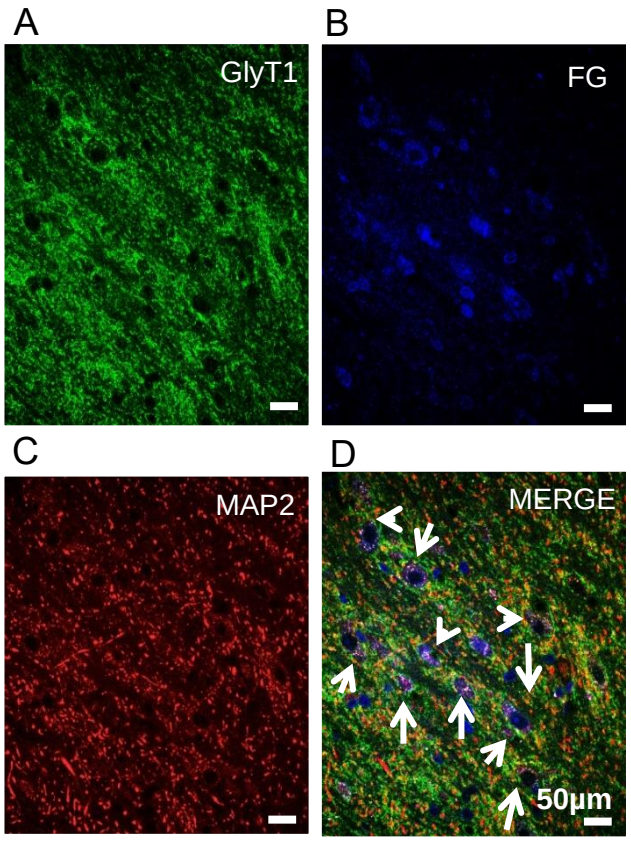

Fig. 4| 63X Confocal images of GlyT1, FG and MAP2. A) Expression of GlyT1 in the SNc B) FG labeled neuronal cell bodies in the SNc C) Neurons and neurites labeled with MAP2 in SNc D) Merged image. Arrows demonstrate GlyT1 membrane expression in FG labeled cell bodies co-localizing with intracellular MAP2.

innervate the striatum (Fig. 7C and 7D). To confirm that the cell bodies in the SNc containing FG correspond to neurons, we proceeded to compare the co-localization of FG and neuronal nuclei (NeuN) antibodies with GlyT1 expression. Confocal images of the SNc show accumulation of FG co-localizing with NeuN-positive neurons, which contain GlyT1 immunoreactivity around the plasma membrane (Fig 8). The confocal images indicate the presence of GlyT1 in glycinergic SNc neurons and disprove the notion that GlyT1 is primarily a glial protein. Consistent with our immunohistochemistry (IHC) data, western blot results demonstrate the presence of GlyT1 and GlyR in the GP (Fig 9). However, the identification of the post-synaptic neurons is still waiting to be investigated. Importantly, these two proteins are devoid in the striatum, consistent with immunofluorescence results.

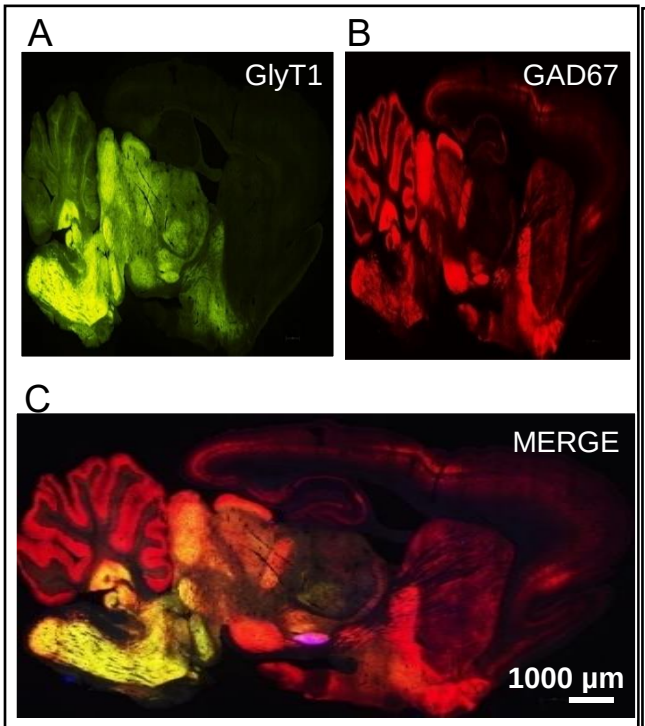

Fig. 5| Co-localization of GlyT1 and GAD67. A) Immunoreactivity of GlyT1 B) Immunoreactivity of GAD67. C) Merged image of GlyT1 and GAD67.

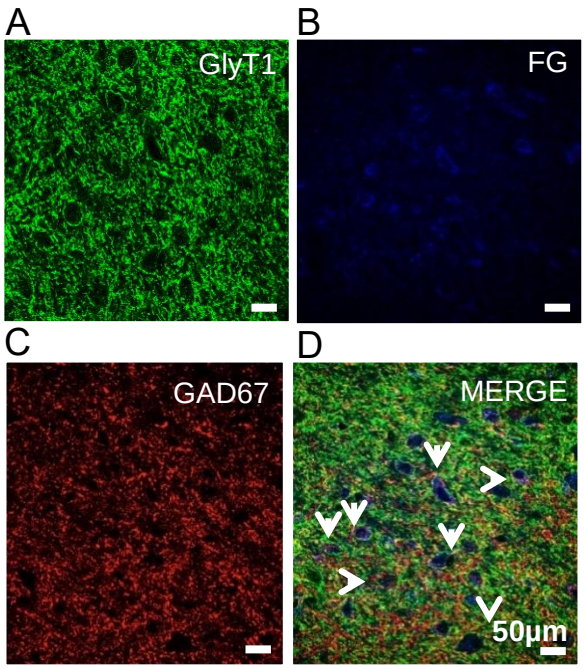

Fig. 6| 63X Confocal images of GlyT1, FG and GAD67. A) GlyT1 immunoreactivity in SNc B) FG labeled neurons in SNc. C) GAD67 expression in SNc. D) Merged image. Arrows indicate co-localizatin of GlyT1, GAD67 and FG.

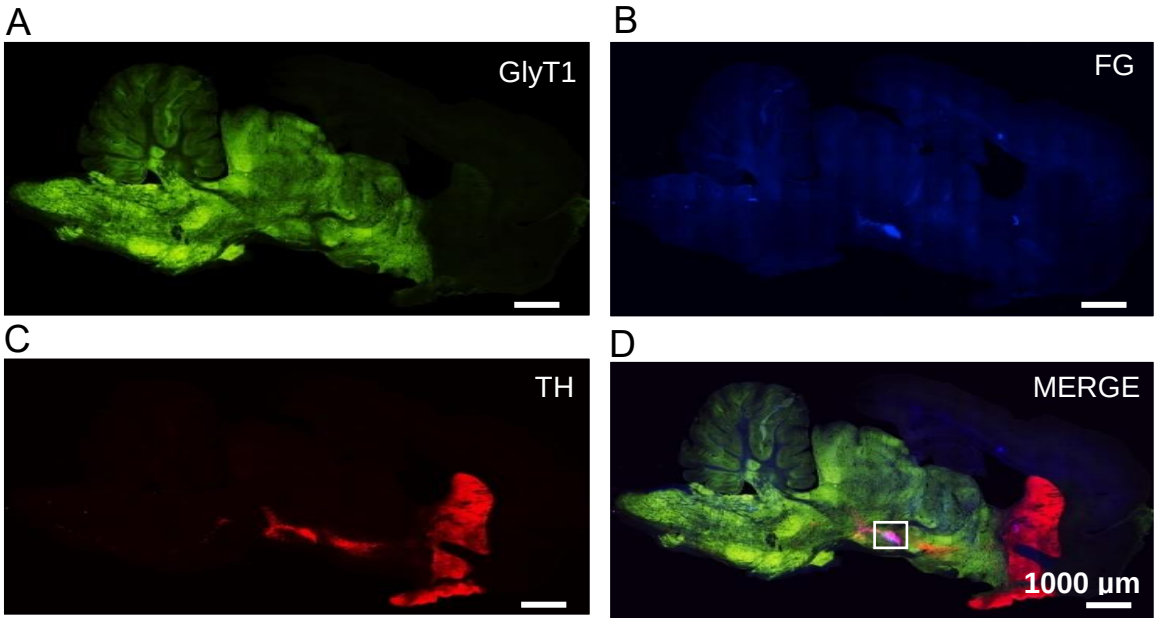

Fig. 7| Localization of GlyT1 and TH. A) Immunoreactivity of GlyT1.B) Retrograde trafficking of FG to the SNc C) Detection of TH D) Merge image.

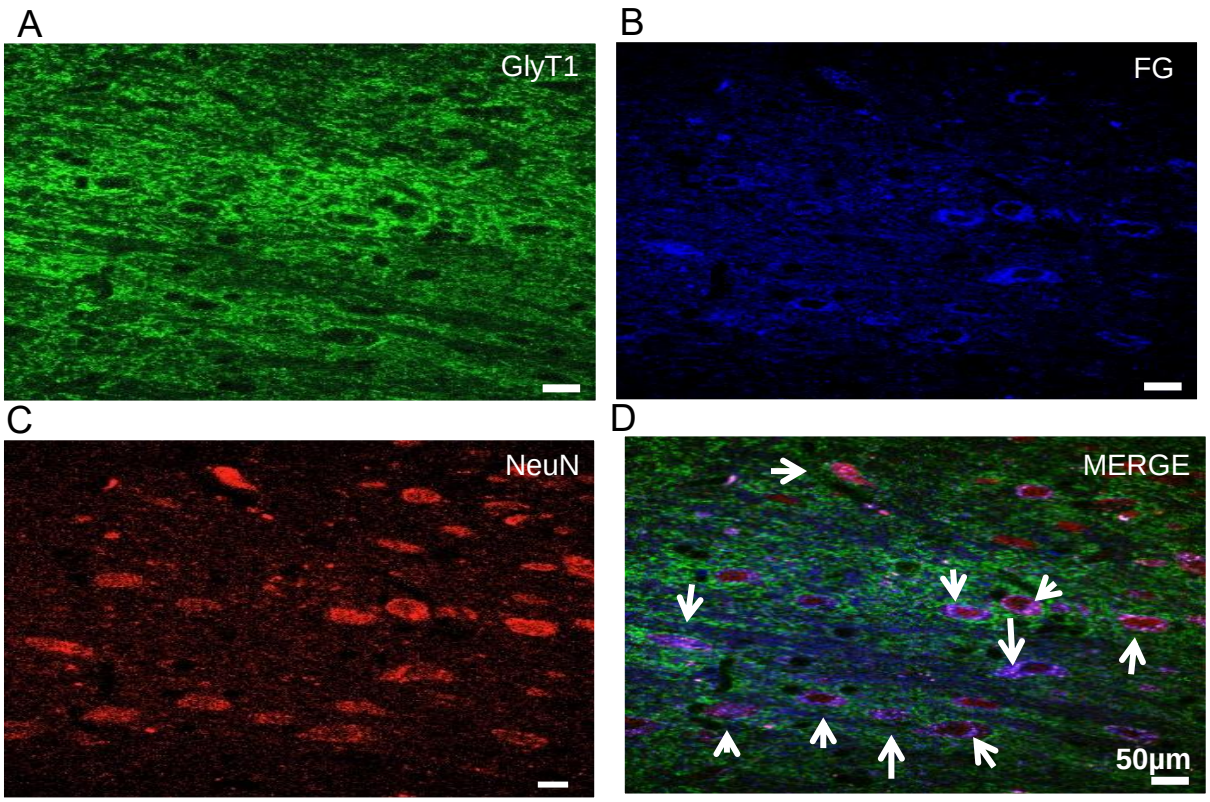

Fig. 8| 63X Confocal images of GlyT1, FG and NeuN. A) of GlyT1 in the SNc B) Neuronal cell bodies labeled with FG in the SNc C) Immunoreactivity of NueN in neuronal cell bodies in SNc D) Merged image. Arrows indicate co-localization of NeuN and FG in cell bodies expressing GlyT1.

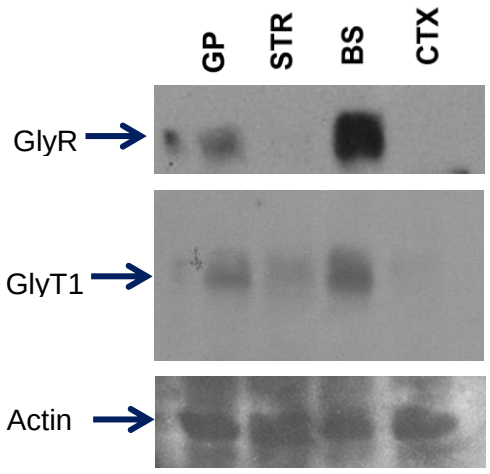

Fig. 9| Western blot of GlyT1 and GlyR. Expression of GlyT1 and GlyR in the GP. GlyT1 and GlyR were not detected in the striatum (STR). The brainstem (BS) was the positive control. The cortex (CTX) was the negative control.

D.1.2B Circuit delineation of nigropallidal neurons using Dextran Red and AAV particles. The anterograde tracer, Dextran Red, was directly delivering (0.4 µl) into the SNc to outline the circuits traveling towards the GP. Nissl staining of the corresponding tissues was used to localize the precise anatomical region of the injection site (Fig 10B). Accurate deposit of the tracer was visualized using Zeiss Observer,

10X objective (Fig 10A). Analysis of the dextran-labeled projections showed axonal circuitry innervating and traversing the GP (Fig 11). Further validation was obtained via AAV particle delivery under the expression of the reporter gene mCherry under the reporter for synapsin. Neuronal transduction of nigropallidal projections was done by injecting 0.150 µl directly into the SNc (Fig 12). The tissue expressing mCherry-positive neuronal cell bodies in the SNc were then subjected to immunohistochemistry. Immunoreactivity of GlyT1 surrounding mCherry-containing cells supports preliminary data and establishes the presence of a glycinergic nigropallidal pathway (Fig 13).

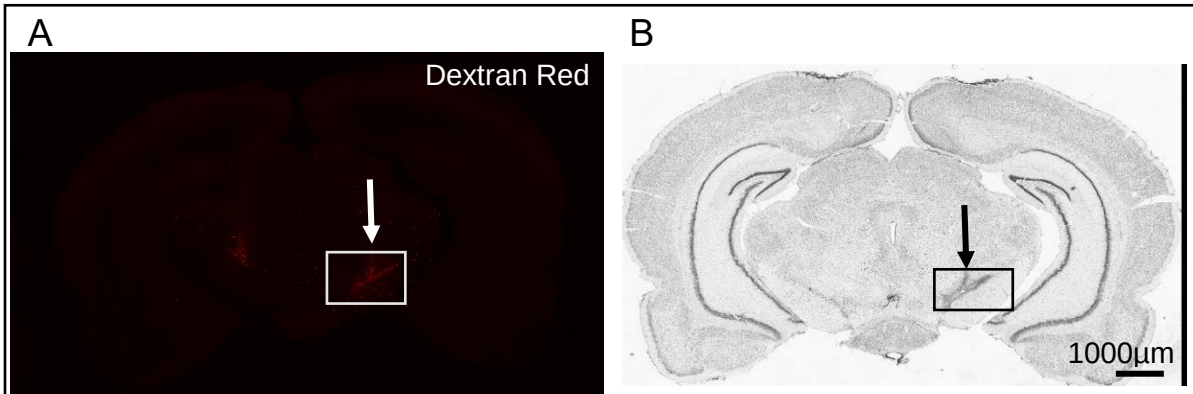

Fig. 10| Delivery of Dextran Red in neurons of the SNc. A) Control image of injections site in SNc. B) Nissl staining of injection site in SNc.

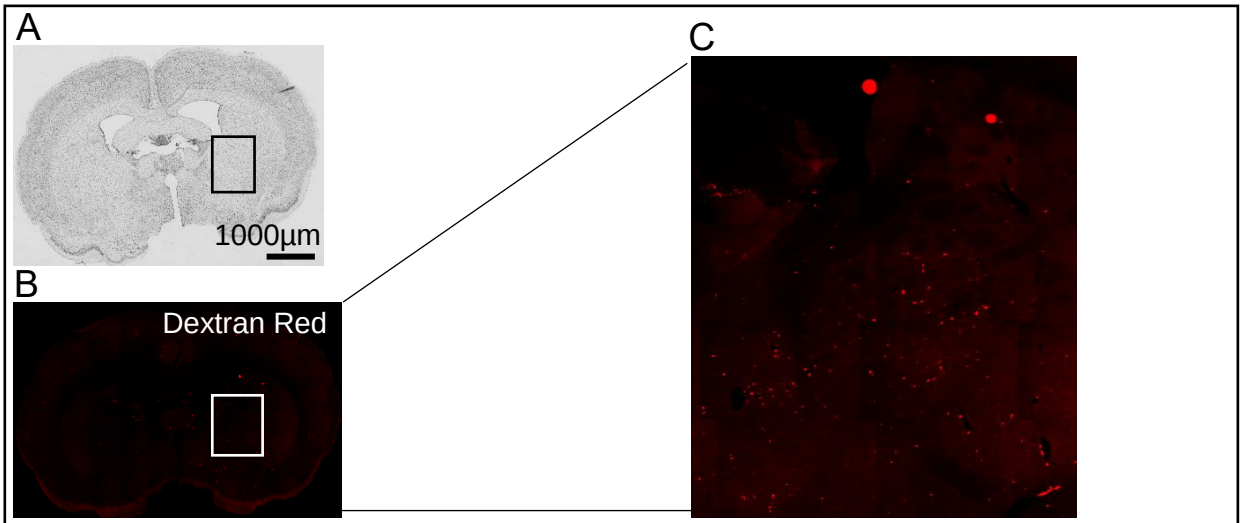

Fig. 11| Dextran Red deposit in the GP. A) Nissl staining of GP containing Dextran Red-positive projections. B) Control image of GP containing Dextran Red-positive projections. C) Close-up image of Dextran Red deposits in GP innervations.

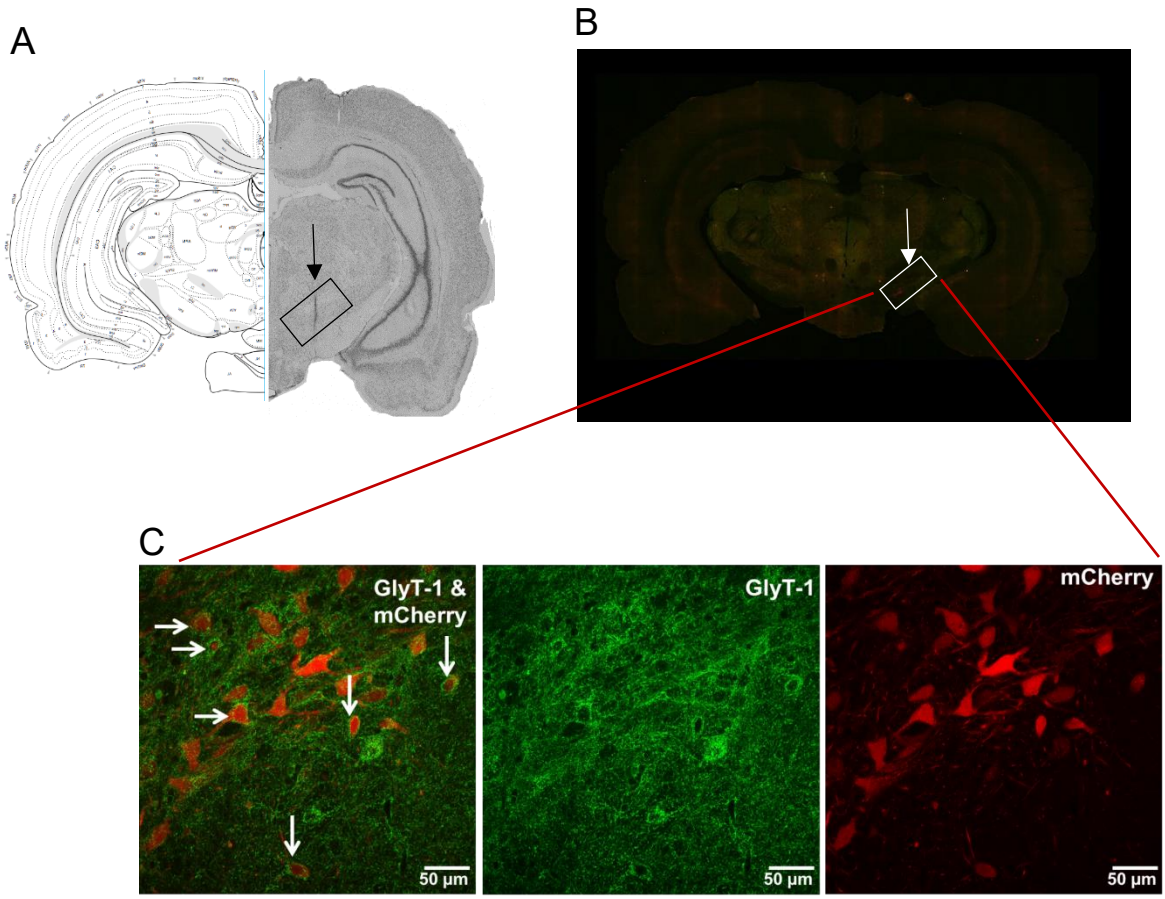

Fig. 12| mCherry deposit into the SNc. A) Injection site of AAV particles into the SNc. B) GlyT1 staining of mCherry-containing tissue. C) Co-localization of Glyt1 and mCherry-positive neurons in the SNc (63X).

D.2 Experimental Design

Identification of glycinergic nigropallidal circuits containing GlyT1 in the basal ganglia using the retrograde tracer FluoroGold, the anterograde tracer Dextran Red and via AAV particle delivery and expression of the reporter gene mCherry. We hypothesized that GlyT1 immunoreactivity in the GP corresponds to glycinergic fibers and that these are projection neurons rather than interneurons; therefore, the cell bodies are outside the GP. To test this hypothesis, we performed neuronal tracing experiments using adult male and female rats and the fluorescent

tracers FG (Invitrogen; 4.0%), Dextran Red (Invitrogen; 10.0%) and AAV particles expressing mCherry. **Stereotaxic surgeries:** Preceding all surgical procedures, all instruments were enclosed in an autoclavable plastic bag (SelfSeal View Pack, Reorder No. 525, Medical action industries Inc.) and were sterilized together with 0.9% saline solution (500 ml) and cotton-tipped applicators (23-400-101, Fisher Scientific). Neuronal tracing experiments were conducted using Sprague-Dawley rats weighing between 270-340 grams. The animals were initially placed in a gas chamber and anesthetized with isoflurane (Vedco, St. Joseph, MO) in oxygen. Once immobilized, the rat's initial weight was recorded, its head was shaved under a biochemical hood, and was then positioned in the stereotaxic device. A heating pad (DC temperature Controller, FHC, Bowdoin, ME) was used throughout the surgical procedure to maintain and monitor body temperature. Cotton-tipped swabs were used to apply moisturizing eye balm (Akwa Tears®, sterile, Akorn, Inc., Lake Forest, IL) to both eyes to help retain moisture. The shaved area was interchangeably sterilized with chlorhexidine disinfectant solution (Chlorhexidine 2% solution, VEDCO, INC., St. Joseph, MO) and alcohol swaps (Webcol®, 2 Ply-Medium, Tyco Healthcare Group L.P., Mansfield, MA). ChloreHex-Q scrub (Chlorhexidine Scrub 2%, VEDCO, INC., St. Joseph, MO) was applied subsequently. A subcutaneous injection of flunixin (1.1 mg/kg Flunazine®, Product No. 1FLU003, Bimeda-MTC Animal Health Inc., Cambridge, ON Canada) was administered before the initial incision to minimize post-operative discomfort. The first set of gloves was then removed and switched out for sterile surgical gloves (SensiCare®, 484401, Medline Industries, Inc., Mundelein, IL). Disposable surgical blades (Sterile Disposable Carbon-Steel Blades,#20, Medline MDS15020, Norwalk, CA) were used to make an incision on the scalp. Four hemostats were utilized to clamp the fascia and expose the skull. Cotton-tipped swabs were dipped in hydrogen peroxide (100%; Fisher Scientific) and used to remove the periosteum and expose the bregma and lambda sutures. Dorsal/ventral measurements of bregma and lambda were adjusted, respectively, to level and align the skull (Illustration 1). Bilateral measurements were then obtained from "The Rat Brain in Stereotaxic Coordinates" by Paxinos and Watson to

determine the drill and injection sites, where a circular opening of approximately 3-4 mm in diameter was made using a drill (Micromotor high-speed drill with drill bits, The Foredom Electric Co., Bethel, CT). The following coordinates were used to carry out the intracranial infusions in the GPe and SNc, respectively: 1) anterior/posterior (AP) -0.96mm, medial/lateral (ML) -2.5mm, +2.5mm, dorsal/ventral (DV) -6.5mm 2) anterior/posterior (AP) -4.92mm, medial/lateral (ML) -2.0mm, +2.0mm, dorsal/ventral (DV) -7.8mm.

Bilateral intracranial injections into the GPe and SNc were performed using glass micropipettes (.5/1, Cat. No. 50611, Stoelting Co. Wood Dale, IL) with tip diameters ranging between 20-25 μm. Capillary action was used to absorb the retrograde tracer FG (4% in water; FluoroChrome, Inc., Englewood, CO) from a piece of parafilm. Once loaded, the glass micropipette was adjusted onto the Picospritzer® III apparatus (Parker Hannifin Corporation, Precision Fluidics Division), which was used to deliver approximately 0.4 μL of the tracer. The site of injection was determined by re-adjusting the AP and ML coordinates. In order to obtain DV measurements, the glass micropipette was placed directly above the orifice. It was subsequently lowered into the target area, where it remained steady for 5 minutes. One pulse of 12 psi in 10 msec was given to deliver the tracer, followed by a 5 minute waiting period (a second pulse was given if necessary). The glass micropipette was slowly retrieved and the incision area was wiped off with saline water and a surgical gauze (2", U.S.P. type VII, sterile, Reorder No. 3322, Dynarex Corporation, Orangeburg, NY). The hemostats were removed and the incision was closed using disposable staples (Weck Visistat® 35R, Teleflex Medical, Research triangle Park, NC). Antibiotic ointment (CURAD Triple Antibiotic Ointment, 10.oz, Medline CUR001231H, Norwalk, CA) was applied onto the wound to prevent it from becoming infected. The rats were relocated into clean post-operative cages lined with surgical bedding and containing food, hydrogel, and snacks and were initially monitored throughout the first four hours to ensure a healthy recovery. A boost-shot

of flunixin was administered 24 hours post-surgery. The animals were monitored daily for 5 consecutive days. All rats remained alive two weeks post-op to allow the fluorescent dye to travel.

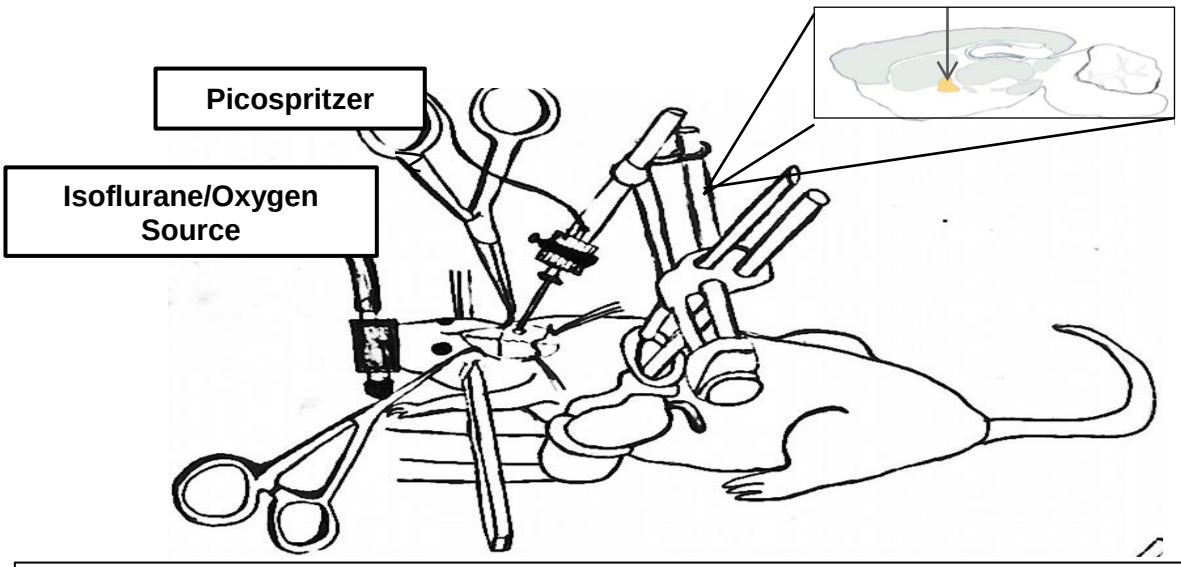

Illustration 1. Stereotactic Surgery Set up and Diagram. Rat is shown after anesthesia, sterilization of the scalp, and leveling of the cranium. The rat is connected to the isoflurane (2.5-3%) and oxygen apparatus. The glass pipette is attached to the adapter, which has tubing that leads to the Picospritzer pressure system. A sagittal view of the rat brain shows the site of injection. The arrow in the inset represent the glass pipette. Kindly donated by Pando M. 2014.

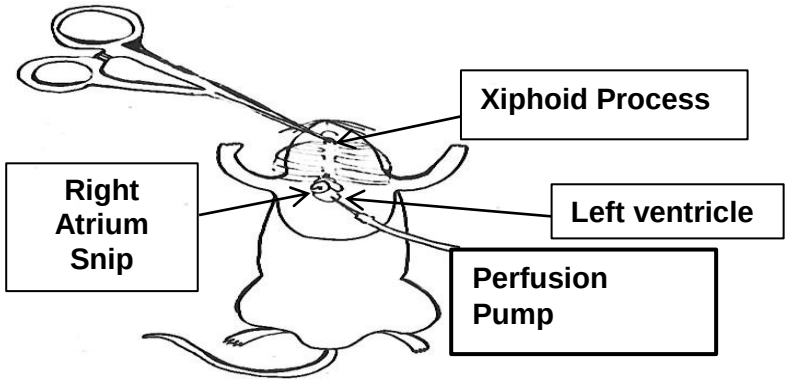

Illustration 2. Perfusion Diagram. An incision is made right below the xiphoid process. The rib cage is flipped upwards using hemostats to clamp the xiphoid process. In the rat, the descending aorta is clamped. Another incision is made in the left ventricle of the heart, where the needle will be placed. A final snip is made in the right atrium to release the flow caused by the perfusion pump. Kindly donated by Pando M. 2014.

E. DISCUSSION

The cumulative data that was gathered suggests that glycinergic neurons are localize within the basal ganglia and that basal ganglia-related functions may be influenced by inhibitory glycinergic neurotransmission. To determine the localization of glycinergic neurons, we performed a series of experiments to differentiate the presence of GlyT1 within neuronal and glial cells, including retrograde and anterograde tracing, immunohistochemistry, and delivery of AAV particles.

The intracranial delivery of FG into the GP, a region that is clearly enriched in GlyT1, allowed FG to traffic to midbrain areas, specifically localized in the SNc. Co-localization of FG with several dopaminergic cell bodies that are labeled with TH is observed within the same area. It is evident that the SNc contains FG in areas mixed with cells that contain TH and GlyT1. Verification of FG deposits in neuronal cell bodies was done via immunohistochemistry and co-localizatin of FG with NeuN and GlyT1. Confocal images of the SNc provided a clear view of the accumulation of FG and NeuN-positive neurons within the cell body, while being surrounded by immunoreactivity for GlyT1. This co-localization demonstrates that the accumulation of the dye is in neurons rather than glial cell. In effect, these results suggest that the injection of FG in the GP is labeling glycinergic neurons in the SNc.

It is described that GlyT1 is found mainly in glial cells, and to demonstrate that GlyT1 is mostly in neuronal bodies in the SNc, we conducted GFAP staining. By contrast, we did not observe many glia cells labeled with GlyT1. These statements disprove the notion that GlyT1 is primarily a glial protein. The confocal images reveal the neuronal presence of GlyT1 surrounding glycinergic SNc neurons. These experiments indicate that the presence of cell containing FG and GlyT1 are neuronal, rather than glial cells. To further confirm that these cells were indeed glycinergic neurons, we continued with an experiment using GlyT1 and MAP2. The neurons

labeled with MAP2 were also diffusely labeled with GlyT1. This further supports the notion of a high number of the neurons in the SNc containing GlyT1 in their membranes.

Given that several groups have reported that some neurons are both GABAergic and Glycinergic (1,9,11), we tested their co-localization in the SNc. To answer this question, we conducted a co-localization immunofluorescent assay of GlyT1 with GAD67. Results showed that regions enriched with GlyT1, specifically the GP, co-localize with GAD67. Importantly, the area that contained FG also contained glycinergic neurons, as well as GAD67- positive neurons. These results suggest a dual phenotype, glycinergic and GAD67 containing glutamate neurons. To further analyze weather the cells containing FG contained GAD67 and GlyT1, we used a higher confocal magnification of the SNc to appreciate the level of co-localization. Images showed that cells containing an increased amount of GlyT1 fluorescence also show GAD67 labeling. This indicates that GAD67 and GlyT1 are present in areas where inhibitory neurons are located. All together, we can conclude that neuronal bodies arising in the SNc, which are innervating the GP, are possibly GABAergic and glyciniergic.

Corroboration of our preliminary results derived from additional tracing experiments. The anterograde dye, Dextran Red, allowed us to delineate the projections arising in the SNc and elucidate the regions of synapse. The reverse outline of SNc pathways demonstrated directionality of the projections towards and past the GP. Furthermore, transduction of neurons in this same region with AAV particles helped us substantiate the presence of glycinergic nigropallidal circuitry within the basal ganglia. Co-localization of the reporter protein mCherry and GlyT1 was obtained via immunofluorescent assays, which provided evidence of co-expression in target neuronal cells.

Altogether, the use of dual antibody staining provided a novel outlook of the expression of GlyT1 within the basal ganglia. In conclusion, our results suggest that neuronal cell bodies and neutrites co-localize with GlyT1 in the SNc, while Glyt1 does not seem to be highly expressed in

glial cells. Also, glycinergic neurons traveling to and though the GP arise from the SNc and contain GlyT1 in their membranes. Importantly, GABAergic neurons in the SNc co-localize with GlyT1, thus suggesting the vesicular co-release of GABA and glycine from pre-synaptic neurons.

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CURRICULUM VITAE

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I obtained my Bachelor's degree from the University of Texas at El Paso (UTEP) in 2009. My early research career started in Dr. Manuel Miranda's lab while I was an undergraduate in 2007. At the time, Dr. Miranda's lab focused on the identification of lysine residues in glycine transporter 1(GlyT1) that were directly involved in protein tagging and degradation. The goal was to study GlyT1 to identify and characterize degradation mechanisms. We conducted site mutagenesis of GlyT1 to change speculative amino acids involved in protein ubiquitination.

While completing my Master's degree in biology, with a concentration in biotechnology, at NMSU, I returned to Dr. Miranda's lab as a volunteer in 2011. I assisted in a study designed to investigate the effects of methamphetamines on dopamine transporter expression in the brain. The research was designed to determine how protein expression differed between experimental rats and control rats.

After finishing graduate school, I started working at UTEP as a part-time research assistant in Dr. Jianying Zhang's lab in 2012. Dr. Zhang's research consists of using proteomic assays such as ELISA, column protein purification and western blotting to identify tumor-associated antigens in human cancer. Part of the process involved performing tests on cancer candidate's blood serum, which analyzed antibody interaction with target antigens. The investigation aimed to identify tumor- associated antigens that may allow for early detection and prediction of the early stages of cancer.

In August 2013, I was accepted to UTEP's graduate program and joined Dr. Manuel Miranda's lab. My project involved the identification of glycinergic circuitry containing GlyT1 in the basal ganglia, of adult rats, using retrograde and anterograde tracing approaches. Retrograde tracing of projections innervating the globus pallidus (GP) was done using the fluorescent dye FluoroGold (FG), and thus localization of the corresponding cell bodies of glycinergic projections.

Circuit delineation of glycinergic nigropallidal neurons was done using the anterograde tracer, Dextran Red, and via Adeno Associated Viral (AAV) particles delivery, and expression of the reporter gene mCherry, into glycinergic nuclei. Additionally, I completed my rotation in Dr. Edward Cataneda's lab in the Psychology Department, where I explored the role that glycinergic neurons in the basal ganglia play in sensorimotor and motor mechanisms in adult rats. To do so, stereotactic surgical procedures were performed to implant unilateral cannulas on the cranium of adult rats, which were directly above our target region, the GP. *In vivo* studies were performed to hinder glycinergic networks in the GP via intracranial infusions of pharmaceuticals targeting GlyT1.